

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

BML  
Sh  
J  
v. 83  
no. 4

April 1989

Volume 83

Number 4

# The Journal of Clinical Investigation

The Journal of clinical  
investigation  
BML Cur Shel  
UNIVERSITY OF CALIFORNIA,  
SAN DIEGO - LIBRARIES  
Received on: 04-14-89  
v. 1- Oct. 1924-

*Published for The American Society for Clinical Investigation, Inc. by The Rockefeller University Press*

EXHIBIT A

## Examples of References

### Journal Articles

#### Authors:

1. Yalow, R. S., and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* 39:1157-1175.

#### More than two authors:

2. Benditt, E. P., N. Ericksen, and R. H. Hanson. 1979. Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 76:4092-4096.

#### In press:

3. Brown, W., and A. Nelson. 1983. Phosphorus content of lipids. *J. Lipid Res.* In press.

### Complete Books

4. Myer, N. B. 1981. *The Biology of Cholesterol and Related Steroids*. Heinemann Medical Books, London. 882 pp.

### Articles in Books

5. Innerarity, T. L., D. Y. Hui, and R. W. Mahley. 1982. Hepatic apoprotein E (remnant) receptor. In *Lipoproteins and Coronary Atherosclerosis*. G. Nosedà, C. Fragaçomo, R. Fumagalli, and R. Paoletti, editors. Elsevier/North Holland, Amsterdam. 173-181.

### Abstract

6. Packman, C. H., S. I. Rosenfeld, and J. P. Leddy. 1981. Inhibition of the C3/C9 steps of complement lysis by a high density lipoprotein (HDL) of human serum. *Fed. Proc.* 40:967a. (Abstr.)

## Figures

Figures must be sorted so that each complete set of figures is submitted in a separate envelope to accompany each copy of the manuscript. Do not submit four copies of each figure in a separate envelope, as this may delay processing of the paper. Mark each envelope with the first author's name and the number of figures enclosed. Each figure must have a label pasted on its back indicating the number of the figure, the names of the authors, and the top of the figure. Do not write on the back of the figures, mount them on cardboard, or scratch or mar them with paper clips. Do not bend figures. Clearly label parts (e.g., A, B) on the figure itself. Give the title in the figure legend, not on the figure. The cost of publishing color prints will be borne by the author. If the prints are out of focus or have been reduced so that the lettering is less than 3 mm high, the original drawings, regardless of size, will be requested upon acceptance of the manuscript. Charts with simple curves will be reduced by the printer to column width (3 inches); plan the size of the lettering so that the height of letters will be about 1½ mm after reduction by the printer. *Electron micrographs and half-tones:* The originals and three sharp sets of glossy prints no larger than 8½ by 11 inches should be sent, each set enclosed in a separate envelope. *Line drawings:* Two sets of glossy prints no larger than 8½ by 11 inches are acceptable if accompanied by two sets of photocopies.

## Legends

*Figure 1.* A short title. In addition, a short explanation should be given in sufficient detail to make the whole intelligible without reference to text (unless similar explanation has been given in another figure).

## Tables

Type double-spaced on sheets separate from the text. Tables should be self contained and self explanatory. Provide a title for the table. Use superscript symbols for footnotes (see Footnotes). Give sufficient explanation and detail in the legend to make the experiment or procedure intelligible without reference to the text. Do not use vertical lines; do not submit photographs of tables.

Tables with different material in the boxheads should be submitted as individual tables (e.g., Tables I, II, III) and not as sections of one table (e.g., Table I A, I B, I C).

## Rapid Publications

Concise papers (5 Journal pages or less) of unusual scientific importance that represent definitive and original studies, completely documented by reference to the literature and by description of the experimental procedures, will be considered in this category. Request for Rapid Publication processing must be stated in the author's covering letter. These papers will be expeditiously reviewed and if accepted will be published at least one month before regular manuscripts accepted on the same date. If rejected, the reasons for rejection and the reviewers' comments will not be sent to the authors. A rejected Rapid Publication may be resubmitted as a regular paper, but will be considered a new manuscript.

One original typescript and 3 copies must be submitted and conform to the following guidelines, in addition to the format for regular manuscripts. The length of each manuscript is determined by the total number of characters in the paper, not the number of pages. The following guidelines have been devised to assist you in preparing your paper.

1. Each line should contain no more than 72 elite or 60 pica characters, including spaces. Each double-spaced page should contain no more than 25 typed lines.
2. The total manuscript should be limited to 550 lines, including partial and blank lines, acknowledgments, footnotes, and references. Count two blank lines before and after every heading.
3. Count each figure or table as 37 lines. To help shorten the manuscript, you should incorporate material into the text rather than present it in figure or tabular form, where possible.
4. Following these guidelines, a double-spaced manuscript that is no longer than 20-22 pages (counting each micrograph as one page and each graph as one-half page and allowing each section to begin on a new page) should fall within acceptable limits. If it is clear that it does not, the authors will be notified.

## Bibliography for Style and Form

1. Council of Biology Editors Style Manual Committee. 1983. *Council of Biology Editors Style Manual: A Guide for Authors, Editors, and Publishers in the Biological Sciences*. Council of Biology Editors, Inc., Bethesda, MD. 5th ed.
2. Webster's Third New International Dictionary of the English Language Unabridged. 1976. P. B. Gove, editor. G. & C. Merriam Company, Springfield, MA.
3. Biosis List of Serials. 1987. BioSciences Information Service of Biological Abstracts, Philadelphia, PA.
4. Collected Tentative Rules and Recommendations of the Commission on Biochemical Nomenclature. 1975. American Society of Biological Chemists, Inc., Bethesda, MD. 2nd ed.

## Addresses

Submit new manuscripts, with a \$50 check or money order per paper for processing to:

Bruce F. Scharschmidt, M.D., Editor  
*The Journal of Clinical Investigation*  
Box 1500, MR IV, Room 108  
University of California, San Francisco  
San Francisco, California 94143-1500  
(415) 476-3924

All correspondence concerning the copyediting and production of accepted manuscripts, as well as requests for permission to reproduce figures or portions of text, should be addressed to:

*The Journal of Clinical Investigation*  
Journals Office  
The Rockefeller University Press  
222 East 70th Street  
New York, New York 10021  
(212-570-8663)

# Transforming Growth Factor- $\beta$

## Murine Glomerular Receptors and Responses of Isolated Glomerular Cells

Karen MacKay, Liliane J. Striker, Joseph W. Stauffer, Toshio Doi, Lawrence Y. Agodoa, and Gary E. Striker  
Metabolic Disease Branch, National Institute of Diabetes and Digestive and Kidney Disease,  
National Institutes of Health, Bethesda, Maryland 20892

### Abstract

Proliferation of resident glomerular cells and the accumulation of mesangial matrix are histologic abnormalities which are observed in the course of many progressive glomerular diseases. We explored the potential regulatory effects of transforming growth factor- $\beta$  (TGF- $\beta$ ) on these processes. We found that cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as isolated intact rat glomeruli possess high-affinity receptors for TGF- $\beta$ . We also found that, although TGF- $\beta$  consistently inhibited the proliferation of glomerular endothelial and epithelial cells, it acted as a bifunctional regulator of mesangial cell proliferation. TGF- $\beta$  significantly increased the production of collagen and fibronectin by glomerular mesangial cells whereas only fibronectin production was augmented in glomerular epithelial cells. The presence of TGF- $\beta$  receptors on intact glomeruli and on each glomerular cell type and the demonstrated responsiveness of these cells to TGF- $\beta$  combine to suggest that potentially important interactions may occur between resident glomerular cells and TGF- $\beta$  in vivo.

### Introduction

Proliferation of resident glomerular cells and/or the accumulation of mesangial matrix constitute the first discernable glomerular morphologic abnormalities in several different renal diseases (1-6). This suggests that dysregulation of factors that control glomerular cell proliferation and mesangial matrix accumulation may play an early and perhaps critical role in the cascade of events leading to a loss of glomerular structural and functional integrity. Recently, several investigators have used cultured glomerular cells to define the responses of individual glomerular cell types to potential regulatory or pathogenic stimuli. These studies demonstrated that mesangial cells exhibit a proliferative response to platelet-derived growth factor (PDGF)<sup>1</sup> (7), epidermal growth factor (EGF) (8), insulin growth factor I (IGF-I) (9), and interleukin 1 (10), and that

heparin species exhibit a negative effect on mesangial cell proliferation (11, 12).

One potentially important modulator of glomerular cell function whose effects on resident glomerular cells remain unexplored is transforming growth factor- $\beta$  (TGF- $\beta$ ). The TGFs were originally defined as peptides that reversibly confer the transformed phenotype on cultured fibroblasts. Isolation and purification of one of the TGFs, TGF- $\beta$ , revealed it to be a disulfide-linked homodimer of 25,000 mol wt that is present in several normal and neoplastic tissues (13). The highest concentration of TGF- $\beta$  is found in platelets which release it during degranulation (14). This peptide has potent effects on the proliferation and differentiation of a variety of cell types as well as on synthesis of extracellular matrix components. Generally TGF- $\beta$  has been found to inhibit the proliferation of cultured cells; however, it may also act as a proliferative stimulus to some of the same cell types under different culture conditions (15, 16). TGF- $\beta$  also acts as a bifunctional regulator of differentiation in that it stimulates the differentiation of normal human bronchial epithelial cells (17) but inhibits the differentiation of myoblasts (18). In addition, TGF- $\beta$  increases the synthesis of collagen by fibroblasts (19) and osteoblasts (20) and increases fibronectin synthesis in a variety of normal and transformed cell types (21). The effects of TGF- $\beta$  on fibronectin synthesis appear to be somewhat dependent on cell type inasmuch as no increase in fibronectin synthesis was noted in aortic smooth muscle cells exposed to TGF- $\beta$  (16).

Since TGF- $\beta$  is present in normal kidney (22) and since platelet and platelet antigens have been found within the glomerulus in a number of renal diseases (23-25), it is likely that glomerular cells are exposed to TGF- $\beta$ . In these studies we demonstrate the presence of high-affinity TGF- $\beta$  receptors on cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as on isolated rat glomeruli. In addition, the effects of TGF- $\beta$  on glomerular cell proliferation and on the production of collagen and fibronectin synthesized by mesangial and epithelial cells are described.

### Methods

**Isolation and identification of mouse glomerular cells.** Glomerular endothelial, mesangial, and epithelial cells were cultured from glomeruli isolated from normal 4-wk-old mice (C57Bl/6J  $\times$  SJL/J) using techniques previously described (26). Pure populations of cells were obtained using the techniques of dilute plating and patch cloning. Endothelial and mesangial cells were maintained in basal medium supplemented with 20% FBS. Basal medium consisted of a 3:1 mixture of MEM/F12 modified with trace elements as described (26), to which glutamine, penicillin, and streptomycin were added. Epithelial cells were maintained in basal medium supplemented with 2% FBS. Cells were subcultured two to three times weekly at a 1:4 split ratio. All

Address reprint requests to Dr. MacKay, Building 10, Room 3N110, National Institutes of Health, Bethesda, MD 20892.

Received for publication 12 January 1988 and in revised form 1 December 1988.

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; IGF-I, insulin growth factor I; PDGF, platelet-derived growth factor; TGF- $\beta$ , transforming growth factor- $\beta$ .

The Journal of Clinical Investigation, Inc.  
Volume 83, April 1989, 1160-1167

well. Petri dishes and flasks were coated with a thin layer of 200 µg/ml human fibronectin (Collaborative Biomedical Products, Inc., Lexington, MA). All experiments were performed on cells between passages 8 and 20. Culture morphology and homogeneity were preserved at all passages tested. Results were verified on a second independently isolated population of each cell type and were qualitatively identical and quantitatively similar.

**Identification of cells.** Immunofluorescence staining was performed as described (26). Immunofluorescence staining for PHMS (Bioscience Products AG, Emmenbrucke, Switzerland) was performed on cells fixed with paraformaldehyde at 4°C for 20 min. Cells were incubated with normal goat serum for 20 min followed by an incubation with a 1:3 dilution of PHMS (27). Cells were then washed with phosphate-buffered saline incubated with biotin-conjugated goat anti-mouse IgG (Tago Inc., Burlingame, CA) washed, incubated with FITC-conjugated streptavidin (Zymed, San Francisco, CA) washed, mounted, and examined.

Assays for alkaline phosphatase and  $\gamma$ -glutamyl-transpeptidase were performed on subconfluent cultures of glomerular epithelial cells as described (26). LLC-PK1 cells (ATCC CRL 1392) served as a positive control. Cells were evaluated for the uptake of acetylated LDL as described (28, 29).

**Binding assay for cells.** Binding of  $^{125}$ I-labeled TGF- $\beta$  to near-confluent monolayers of individual glomerular cell types was determined as described (30, 31). For these assays  $5-7 \times 10^4$  cells were plated per well in 24-well cluster plates in basal medium supplemented with 10% FBS for endothelial and mesangial cells and 2% FBS for epithelial cells. Purified TGF- $\beta$  was isolated from human platelets and iodinated as described (30).

The decrease in TCA precipitability of the labeled ligand after the 2-h incubation with the monolayers was  $\leq 4\%$ . Values for free and bound radioactivity were determined on duplicate wells which typically differed by  $\leq 7\%$ .

For displacement experiments, total and nonspecific binding were allowed to reach equilibrium during a 90-min incubation at 22°C. Labeled ligand was added at a concentration of 50 pM. Nonspecific binding was determined in wells containing a 100-fold excess of unlabeled ligand. After the 90-min incubation (time zero) binding media were removed, the cells were washed twice with fresh binding medium, and the medium was replaced with binding medium containing 5 nM unlabeled TGF- $\beta$ . Displacement was determined as the decrement in specifically bound counts over time compared to the amount of specific binding present at time zero. 80% of the displaced counts were precipitable with TCA after a 3-h displacement period.

**Binding assay on intact glomeruli.** Female Sprague-Dawley rats weighing approximately 250 g were anesthetized with a subcutaneous injection of Innovar-Vet (Pitman-Moore Inc., Washington Crossing, NJ). Glomeruli were isolated (using 190- and 74-µm sieves from E-C Apparatus Corp., St. Petersburg, FL) from kidneys which had been flushed free of blood with binding buffer. Isolated glomeruli were incubated with constant rotation in binding buffer for 1 h at 37°C. Glomerular number and purity were determined by counting three separate 2-ml aliquots of this glomerular suspension using a scored counting chamber under phase contrast microscopy. Counts consistently differed by  $\leq 15\%$ . Glomerular preparations were 90-95% pure and contained over 90% decapsulated glomeruli. Aliquots containing 150 glomeruli were centrifuged (25 g for 1 min). Medium was replaced with 0.5 ml of binding buffer containing varying amounts of  $^{125}$ I-labeled TGF- $\beta$  with or without 10 nM unlabeled TGF- $\beta$ . After a 2-h incubation with constant rotation at room temperature the glomeruli were briefly centrifuged, the media removed, the glomeruli washed four times in wash buffer and the glomerular pellet was counted in a gamma counter. The binding media and the first wash were pooled and counted to determine the free ligand concentration.

TCA precipitability of the labeled ligand incubated with glomeruli decreased by  $\leq 2\%$  during the assay. Values for free and bound radioactivity were determined on duplicate samples. These values typically

differed by  $\leq 7\%$ . Time course and displacement assays for glomerular binding were performed as described above.

**Effect of TGF- $\beta$  on FBS stimulated glomerular cell proliferation.** On day zero,  $5 \times 10^4$  cells were plated in each well of 24-well cluster plates in basal medium supplemented with 2% FBS. After 6-8 h of incubation TGF- $\beta$  was added to the wells at the indicated concentrations. Cells in three to four replicate wells were trypsinized and counted using an ELZONE<sup>®</sup> electronic particle counter (Particle Data Inc., Elmhurst, IL).

**Thymidine incorporation assays.** Cells were plated at  $2.5-5 \times 10^4$  per well in 24-well plates in medium containing 10% (endothelial and mesangial cells) or 2% FBS (epithelial cells). At 24 h cells were washed and the medium changed to serum-free medium. This medium was replaced every 24 h until no increase in cell number over 24 h was noted (this required 36 h for epithelial cells, and 72 h for mesangial and endothelial cells). Longer periods of serum starvation resulted in loss of cell viability. This resulted in basal [ $^3$ H]thymidine incorporation of 5,000-10,000 cpm during the 12-h labeling period. FBS or ligands (EGF, IGF-I [AMGen Biologicals, Thousand Oaks, CA], or partially purified PDGF [Collaborative Research, Bedford, MA] were added in fresh serum-free medium containing 0.1% BSA and incubation continued for 10 h after which 1 µCi per well of [ $^3$ H]thymidine (Amersham Corp., Arlington Heights, IL) was added. After an additional 12-h incubation the monolayers were washed with PBS, precipitated and washed twice with 10% TCA, and then solubilized in 1.0 N NaOH. The contents of each well and one PBS wash were neutralized with HCl and counted in a liquid scintillation counter. Results are expressed as the mean of duplicate determinations. Duplicates consistently differed by  $\leq 10\%$ .

**Density-dependent proliferation of cells in response to TGF- $\beta$ .** 2, 4, 6, 8, or  $10 \times 10^4$  cells were plated in two 24-well cluster plates in basal medium supplemented with 2% FBS. 8 h later TGF- $\beta$  was added to a final concentration of 10 pM to one of the plates. Cell number in triplicate or quadruplicate wells was determined after 36 h as described above.

**Collagen production.** Mesangial cells were plated at  $2 \times 10^5$  cells per well in six-well cluster plates in basal medium supplemented with 10% FBS. After 24 h the cells were washed with basal medium and incubated in basal medium with 0.2% FBS. After 12 h medium was again changed to fresh medium containing 0.2% FBS to which varying concentrations of TGF- $\beta$  was added. After an additional 12 h medium was changed to proline-free medium with 0.2% FBS, the indicated amount of TGF- $\beta$ , 25 mM ascorbic acid (Sigma Chemical Co., St. Louis, MO), and 5 µCi of L-[2,3- $^3$ H]proline (Amersham Corp.). After 5 h the medium and cells were harvested and assessed for collagen production as described (20, 32) using chromatographically purified bacterial collagenase (Advance Biofactures, Lynbrook, NY). Collagen production was determined on medium and monolayers collected from triplicate wells which were processed separately. Cell number was determined on duplicate wells at each TGF- $\beta$  concentration tested. Monolayers contained  $< 10\%$  of total collagenase sensitive protein and exhibited no change in response to TGF- $\beta$ .

Collagen production in near confluent cultures of glomerular epithelial cells was assessed as described for mesangial cells except that the epithelial cells were plated in 2% rather than 10% FBS.

**Fibronectin synthesis.** Cells were plated and medium changes performed as described for collagen production assays. After 12 h of incubation with different concentrations of TGF- $\beta$ , the medium was changed to cysteine-free medium containing 0.2% FBS, the indicated amount of TGF- $\beta$ , and 20 µCi of [ $^{35}$ S]cysteine (Amersham Corp.). The cells were labeled for 3 h, after which the medium and cell layers were processed as described (21) except that rabbit anti-mouse fibronectin (Biomedical Technologies, Inc., Stoughton, MA) was used for the immunoprecipitation studies. Cell counts were determined on duplicate wells at each concentration of TGF- $\beta$ . The concentration of medium and cell lysates was adjusted for cell counts before immunoprecipitation was performed.

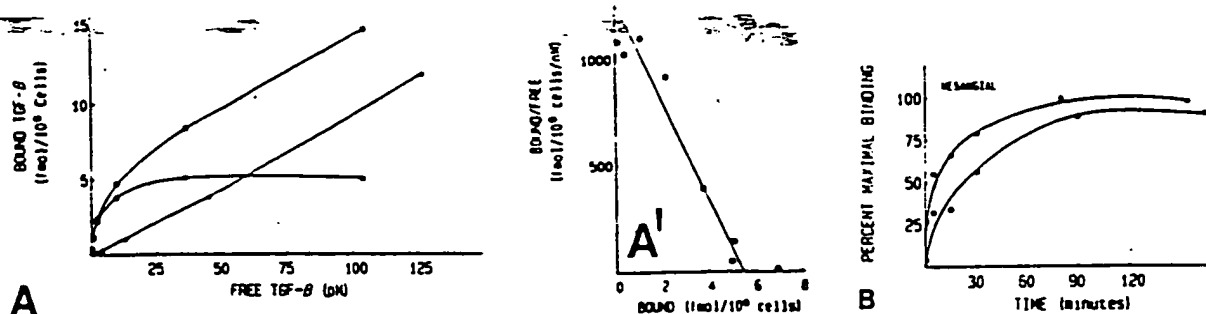


Figure 1. (A) TGF- $\beta$  binding to mesangial cells as a function of free ligand concentration. (■) Specific binding is the difference between the (□) total and (○) nonspecific binding. Nonspecific binding was determined in the presence of 10 nM unlabeled TGF- $\beta$ . Each point is the mean of two determinations that generally differed by  $\leq 7\%$ . (A') Scatchard plot of TGF- $\beta$  binding to mesangial cells. (B) Time and temperature dependence of TGF- $\beta$  binding to glomerular mesangial cells. Percentage of maximal specific binding at (■) 22°C and (○) 4°C are expressed as a function of time after addition of the labeled ligand. <sup>125</sup>I-labeled TGF- $\beta$  was added at a concentration of 50 pM to all wells. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand. Similar results were obtained for glomerular endothelial and epithelial cells.

## Results

**Identification of glomerular cells.** Each glomerular cell type was identified by a combination of several characteristic features. Glomerular endothelial cells grew as a monolayer of cobblestone appearing cells at confluence. By immunofluorescence staining all cells were positive for factor VIII-related antigen and negative for cytokeratin, had a distribution of actin about the cell periphery, and displayed uptake of acetylated LDL. Glomerular mesangial cells had elongated cell processes at subconfluence and appeared as elongated cells aligned in parallel arrays at confluence. The cells did not stain for factor VIII-related antigen or cytokeratin, did not evidence uptake of acetylated LDL, and were able to grow in medium containing D- rather than L-valine. These cells had actin filaments running parallel to the long axis of the cell. Epithelial cells grew in patches of cobblestone-appearing cells at subconfluence which coalesced at confluence. The cells did not stain for factor VIII-related antigen and had a peripheral distribution of actin filaments. Epithelial cells, unlike endothelial and mesangial cells, stained with anti-cytokeratin antibody in what appeared to be a cell cycle-dependent manner. Cells that stained for cytokeratin appeared to either be in the process of or to have just completed cell division. All epithelial cells also stained with a monoclonal antibody directed against a human visceral glomerular epithelial cell sialoprotein which shares properties with rat podocalyxin (27). Contamination of glomerular epithelial cells by proximal tubular cells was further excluded by their lack of alkaline phosphatase or gamma glutamyl transpeptidase activity (activity expected in proximal tubular cells) in histochemical assays (26).

**TGF- $\beta$  binding studies.** High-affinity receptors for TGF- $\beta$  were present on all three glomerular cell types. A representative binding curve and Scatchard analysis are shown for glomerular mesangial cells in Fig. 1 A. Similar binding curves were found for glomerular endothelial and epithelial cells. The results of Scatchard analysis for glomerular endothelial, mesangial, and epithelial binding studies are provided in Table I. Each glomerular cell type possessed TGF- $\beta$  receptors of similar

number and affinity. TGF- $\beta$  binding to glomerular cells was time and temperature dependent as shown for mesangial cells in Fig. 1 B. Similar results were obtained for glomerular endothelial and epithelial cells.

Specifically bound TGF- $\beta$  could be displaced from glomerular cells by addition of an excess of unlabeled ligand. For each glomerular cell type  $\sim 50\%$  of specifically bound ligand at equilibrium could be displaced during a 3-h incubation with a 100-fold excess of unlabeled ligand. 80% of the displaced counts were precipitable with TCA at the end of the 3-h incubation.

Freshly isolated rat glomeruli were assayed to determine whether TGF- $\beta$  receptors were also present in vivo. Rat glomeruli were used instead of mouse so as to obtain a glomerular preparation containing a minimal amount of contaminating tubular fragments. Binding curves and Scatchard analysis for rat glomerular TGF- $\beta$  binding are presented in Fig. 2 A. TGF- $\beta$  binding to glomeruli was time dependent as shown in Fig. 2 B. TGF- $\beta$  binding to glomeruli at 4°C gave erratic results, perhaps because of impaired diffusion within the glomeruli at this temperature. After binding equilibrium was reached 75% of the specifically bound TGF- $\beta$  could be displaced by the addition of a 100-fold excess of unlabeled ligand

Table I. Properties of TGF- $\beta$  Receptors on Isolated Glomerular Cells

| Cell type   | $K_d$<br>pM | n/cell |
|-------------|-------------|--------|
| Endothelial | 5           | 3,700  |
| Mesangial   | 5           | 3,900  |
| Epithelial  | 9           | 9,900  |

Results are expressed as means of two separate experiments.  $K_d$  differed by 1 pM or less in replicate experiments. Number of receptors per cell differed by  $\leq 15\%$  in replicate experiments.

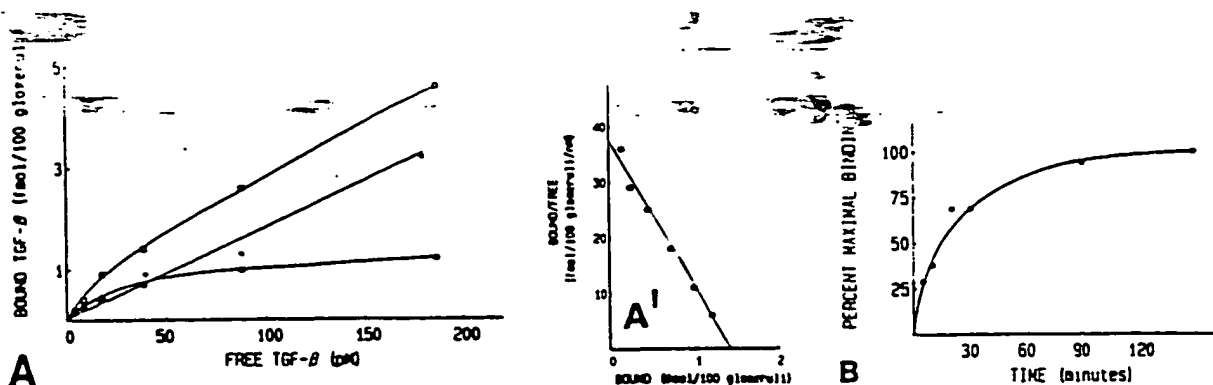


Figure 2. (A) TGF- $\beta$  binding to rat glomeruli as a function of free ligand concentration. (■) Specific binding is the difference between (□) total and (△) nonspecific binding. Each point is the mean of two determinations that generally differed by  $\leq 7\%$ . (A') Scatchard plot of TGF- $\beta$  binding to rat glomeruli. (B) Time course of TGF- $\beta$  binding to rat glomeruli. Each point represents the percentage of maximal specific binding. Experiments were performed at 22°C with 50 pM  $^{125}$ I-labeled TGF- $\beta$  with or without 100-fold excess of unlabeled ligand.

during a 3-h displacement period. 90% of the displaced counts could be precipitated with TCA.

**Effects of TGF- $\beta$  on glomerular cell proliferation.** The effect of TGF- $\beta$  on the proliferation of glomerular cells stimulated by 2% FBS is shown in Fig. 3. TGF- $\beta$  inhibited the proliferation of glomerular endothelial, mesangial, and epithelial cells. Maximum inhibition was present at a concentration of 10 pM TGF- $\beta$  in all cell types. The inhibition of proliferation was not a reflection of impairment of plating efficiency induced by TGF- $\beta$  since cell counts on day 1, 12 h after addition of TGF- $\beta$ , were not different between wells with or without TGF- $\beta$ .

To determine the interaction of TGF- $\beta$  with other mitogens, [ $^3$ H]thymidine incorporation was examined in subconfluent serum-starved cells stimulated by EGF, IGF-I, or PDGF. As shown in Fig. 4, TGF- $\beta$  inhibited the increase in [ $^3$ H]thymidine incorporation induced by EGF, IGF-I, and PDGF in all three glomerular cell types.

The proliferative response of glomerular mesangial cells to TGF- $\beta$  was more complex than that for glomerular endothelial and epithelial cells. During the course of the collagen synthesis experiments described below we consistently observed that mesangial cells at confluence or near confluence proliferated in response to the addition of TGF- $\beta$ . As shown in Fig. 5, proliferation of mesangial cells plated at low density (as they were for the experiments described above) was inhibited by the addition of TGF- $\beta$  while cells at higher density exhibited a proliferative response to TGF- $\beta$ . Neither glomerular endothelial nor glomerular epithelial cells evidenced a proliferative response to TGF- $\beta$  when tested under identical conditions.

TGF- $\beta$  induced a morphologic change in serum-depleted mesangial cells. Mesangial cells characteristically have an elongated, spindle shape when grown with high concentrations of serum. At low serum concentrations (< 5% FBS) they assumed a more cuboidal morphology. The addition of TGF- $\beta$  to these cells resulted in the prompt (< 12 h) return to the typical elongated mesangial cell morphology (Fig. 6). This morphologic alteration was noted in both sparse and near confluent cells.

**Effects of TGF- $\beta$  on glomerular collagen and fibronectin production.** Along with its effects on proliferation and mor-

phology, TGF- $\beta$  increased the production of collagen and fibronectin synthesized by glomerular mesangial cells. The addition of TGF- $\beta$  resulted in a twofold increase in the amount of collagenase sensitive protein in the medium of mesangial cells (Table II). TGF- $\beta$  also significantly increased the production of fibronectin by mesangial cells (Fig. 7). TGF- $\beta$  failed to augment collagen production by glomerular epithelial cells but did cause a substantial increase in epithelial cell fibronectin production (Fig. 8).

## Discussion

The present study reports the presence of receptors for TGF- $\beta$  on intact glomeruli and on isolated glomerular endothelial, mesangial, and epithelial cells. The effects of TGF- $\beta$  on glomerular cell proliferation and extracellular matrix production were also explored.

We found that cultured mouse glomerular endothelial, mesangial, and epithelial cells possess TGF- $\beta$  receptors of similar high affinity ( $K_d = 5, 5, 9$  pM, respectively). Furthermore, high-affinity TGF- $\beta$  receptors were also demonstrated on isolated rat glomeruli. The receptors on intact glomeruli were of somewhat lower affinity than those of cultured mouse cells ( $K_d = 20, 24, 37$  pM in three separate experiments).

The significance of the finding of TGF- $\beta$  receptors on glomeruli was that, heretofore, TGF- $\beta$  receptors have only been demonstrated on isolated cells. The presence of TGF- $\beta$  receptors on glomeruli adds significant support to the hypothesis that TGF- $\beta$  is a potent modulator of cell behavior in vivo as well as in vitro. Further studies will be required to determine whether the differences in TGF- $\beta$  receptor affinity in cultured glomerular cells and isolated glomeruli reflect species differences or alterations in TGF- $\beta$  receptor affinity in the cultured cells.

Since proliferation of resident glomerular cells and accumulation of mesangial matrix material are prominent histologic findings in several progressive glomerular diseases (1-6), these studies focused on the effects of TGF- $\beta$  on these processes. TGF- $\beta$  consistently inhibited the proliferation of glomerular endothelial and epithelial cells. TGF- $\beta$  inhibited serum stimulated proliferation of sparse and densely plated

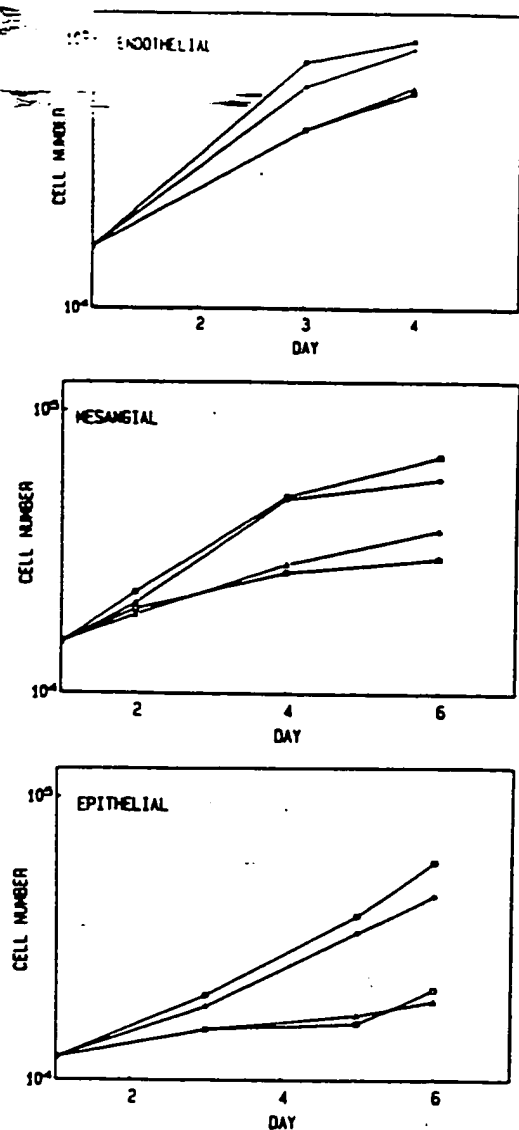


Figure 3. Effect of TGF- $\beta$  on proliferation of glomerular cells. Cells were plated in basal medium supplemented with 2% FBS on day 0. 6–8 h later TGF- $\beta$  was added at a concentration of ( $\square$ ) 0, ( $\diamond$ ) 1, ( $\triangle$ ) 10, or ( $\circ$ ) 100 pM. Cells counts are the mean of triplicate or quadruplicate determinations, which differed by < 10%. The final difference in cell number between cells with and without 10 pM TGF- $\beta$  was statistically significant ( $P < 0.05$ ) for all cell types as determined by unpaired  $t$  test.

endothelial and epithelial cells and diminished or abolished the stimulation of DNA synthesis induced by EGF, IGF-I, and PDGF.

The effects of TGF- $\beta$  on mesangial cells were complex. TGF- $\beta$  acted as a bifunctional regulator of mesangial cell proliferation. In sparsely plated cells TGF- $\beta$  diminished or ablated the proliferative response of mesangial cells to FBS, EGF, IGF-I, and partially purified PDGF. However, TGF- $\beta$  stimulated the proliferation of more densely plated mesangial cells. In addition, TGF- $\beta$  increased the production of both collagen and fibronectin in mesangial cell culture medium and increased the production of fibronectin by glomerular epithelial

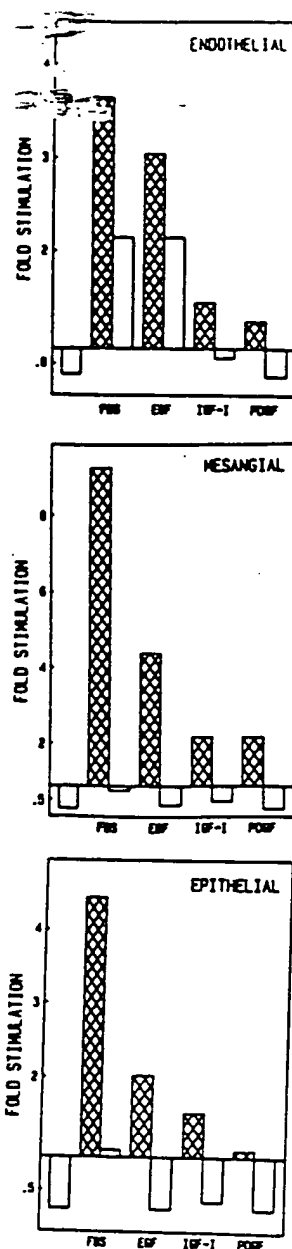


Figure 4. Effect of 10 pM TGF- $\beta$  on [ $^3$ H]thymidine incorporation in subconfluent serum-starved cells stimulated with 2% FBS, 30 ng/ml EGF, 20 ng/ml IGF-I, or 2 half-maximal U/ml partially purified PDGF. Basal incorporation was defined as that which occurred during the 12-h labeling period in cells incubated in medium containing only 0.1% BSA. The open bar on the left of each graph indicates the decrease in basal counts in cells incubated in the presence of 0.1% BSA and 10 pM TGF- $\beta$ . The pair of bars above each indicated ligand represent the fold increase in basal counts induced by the ligand with (open bars) or without (hatched bars) 10 pM TGF- $\beta$ . Each bar represents the mean of duplicate determinations which differed by < 10%.

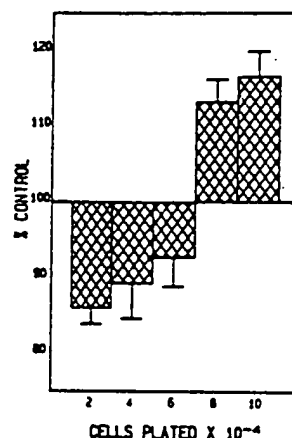


Figure 5. Density-dependent proliferation of mesangial cells in response to TGF- $\beta$ . Cells were plated as described and TGF- $\beta$  was added 8 h later. After a 36-h incubation in the presence or absence of TGF- $\beta$ , cell number was determined by trypsinizing and counting the contents of each well. The cell count in each TGF- $\beta$  treated well was compared with the count determined in the identical well of the non-TGF- $\beta$ -treated plate. Data are expressed as the mean  $\pm$  SEM of triplicate or quadruplicate determinations. By unpaired  $t$  test  $P < 0.05$  for cells plated at 2 and  $10 \times 10^4$ /well.



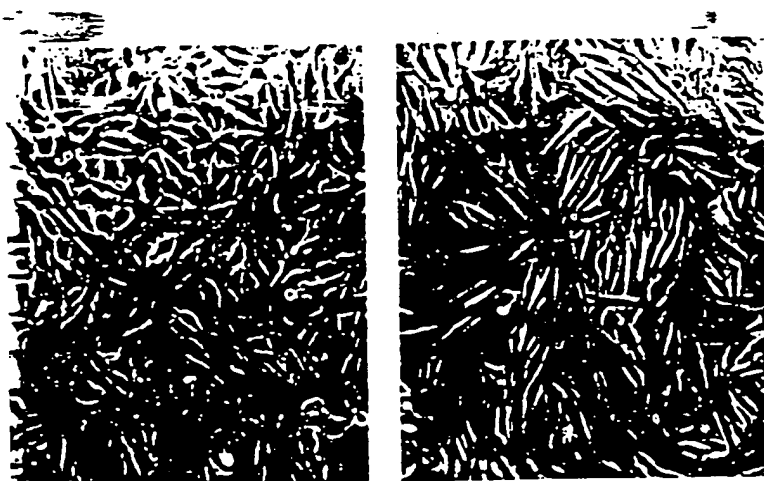


Figure 6. Alterations in mesangial cell morphology in response to TGF- $\beta$ . Mesangial cells were plated and medium changes performed as described for the collagen production assay in Methods. (A) Cells incubated without TGF- $\beta$ . (B) Cells incubated with 10 pM TGF- $\beta$ .

cells. We also found that TGF- $\beta$  restored the elongated morphology considered typical of cultured mesangial cells. This morphologic alteration and the density-dependent effects on mesangial cell proliferation are analogous to results obtained in aortic smooth muscle cells exposed to TGF- $\beta$  (16).

TGF- $\beta$  could be provided to the glomerulus in vivo from a number of sources. One likely source is platelets which release TGF- $\beta$  during degranulation (14). Platelets are a particularly rich source of TGF- $\beta$  as they contain approximately 100-fold more TGF- $\beta$  than other cells or tissues examined to date. A number of factors combine to indicate a role of platelets or platelet products in the pathogenesis or glomerular lesions. Platelets, platelet antigens, or platelet-derived inflammatory products have been identified within the glomerulus in both immune and nonimmune mediated glomerular diseases (23-25). In addition, agents expected to decrease glomerular deposition of platelets and platelet aggregation have been shown to have a beneficial effect on the progression of the glomerulosclerosis observed in animals subjected to partial nephrectomy (33-35) and in patients with membranoprolifer-

ative glomerulonephritis (36). Activated macrophages (37) and lymphocytes (38) represent other potential sources of TGF- $\beta$  within the glomerulus. The presence of TGF- $\beta$  receptors on each glomerular cell type and on isolated glomeruli, the multiple potential sources of TGF- $\beta$  within the glomerulus, and the demonstrated responsiveness of cultured glomerular cells to TGF- $\beta$  combine to suggest that potentially important interactions may occur between resident glomerular cells and TGF- $\beta$  in vivo. The nature of these in vivo interactions is difficult to precisely define since a cell's response to TGF- $\beta$  may be determined in large measure by ambient conditions. In vivo studies will be necessary to clarify the reactions of resident glomerular cells to TGF- $\beta$  under normal and pathologic circumstances. These results indicate, however, that TGF- $\beta$  may play a substantive role in the regulation of processes which appear critical to the initiation of progressive glomerular diseases, i.e.,

Table II. Effect of TGF- $\beta$  on Mesangial Cell Collagen Accumulation

| TGF- $\beta$ | Non-collagen protein            | Collagenase-sensitive protein   |
|--------------|---------------------------------|---------------------------------|
| pM           | cpm $\times 10^{-3}/10^6$ cells | cpm $\times 10^{-3}/10^6$ cells |
| 0            | 2.8<br>(0.30)                   | 6.0<br>(2.98)                   |
| 10           | 3.2<br>(0.34)                   | 12.1<br>(3.85)                  |
| 50           | 3.9<br>(0.42)                   | 13.4<br>(3.85)                  |
| 100          | 3.7<br>(0.45)                   | 12.8<br>(3.74)                  |

Results are expressed as the mean and (standard deviation) of nine determinations assessed in three separate experiments in which triplicate wells at each concentration were processed separately. Using the unpaired *t* test for non-collagen protein 0 vs. 10 pM,  $P = 0.02$ ; for 0 vs. 50 and 100 pM,  $P < 0.0001$ . For collagenase-sensitive protein,  $P < 0.001$  for 0 vs. 10, 50, and 100 pM TGF- $\beta$ .

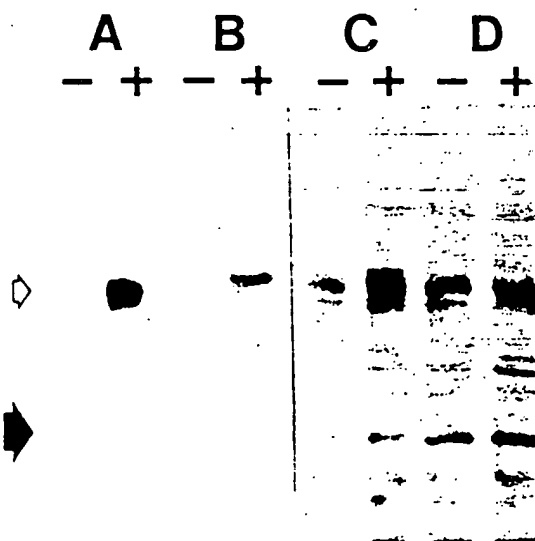


Figure 7. Effect of TGF- $\beta$  on accumulation of fibronectin synthesized by glomerular mesangial cells. Medium (A, B) and monolayers (C, D) were prepared as described in Methods. Cultures were incubated with (+) or without (-) 50 pM TGF- $\beta$ . Preparations were precipitated either with gelatin sepharose (A, C) or with anti-fibronectin antibody followed by protein A sepharose (B, D).

A B



Figure 8. Effect of TGF- $\beta$  on production of fibronectin by glomerular epithelial cells. Cultures were incubated with (+) or without (-) 50 pM TGF- $\beta$ . Preparations from the cell monolayer were precipitated either with gelatin sepharose (A) or with anti-fibronectin antibody followed by protein A sepharose (B). Minimal amounts of fibronectin were detected in the culture medium.

proliferation of mesangial cells and accumulation of mesangial matrix.

## Acknowledgments

TGF- $\beta$  and  $^{125}$ I-labeled TGF- $\beta$  were the generous gifts of Dr. Lalage M. Wakefield. The authors thank Sharon Elliot who isolated a second population of glomerular endothelial cells utilized to confirm our results.

Dr. MacKay is a recipient of an Individual National Research Service Award DK07843-02.

## References

1. Mauer, S. M., M. W. Steffes, E. N. Ellis, D. E. R. Sutherland, D. M. Brown, and F. C. Goetz. 1984. Structural-functional relationships in diabetic nephropathy. *J. Clin. Invest.* 74:1143-1155.
2. Schwartz, M. M., and E. J. Lewis. 1985. Focal segmental glomerular sclerosis: the cellular lesion. *Kidney Int.* 28:968-974.
3. Heptinstall, R. H. 1983. The nephrotic syndrome. In *Pathology of the Kidney*, R. H. Heptinstall, editor. Little, Brown & Co., Boston/Toronto. 676-696.
4. Donadio, J. V., and K. E. Holley. 1982. Membranoproliferative glomerulonephritis. *Semin. Nephrol.* 2:214-227.
5. Sterzel, R. B., and R. B. Pabst. 1982. The temporal relationship between glomerular cell proliferation and monocyte infiltration in experimental glomerulonephritis. *Virchows Arch. B. Cell Pathol.* 38:337-350.
6. Yoshikawa, N., K. Iijima, K. Maehara, S. Yoshiara, K. Yoshiya, T. Matsuo, and S. Okada. 1987. Mesangial changes in IgA nephropathy in children. *Kidney Int.* 32:585-589.
7. Shultz, P. J., P. E. DiCorleto, B. J. Silver, and H. E. Abboud. 1988. Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am. J. Physiol. (Renal Fluid Electrolyte Physiol.)* 245:F674-684.
8. Abboud, H. E., C. Saunders, and T. C. Knauss. 1987. Effect of epidermal growth factor (EGF) on cultured mesangial cells. *Kidney Int.* 31:184a. (Abstr.)

9. Conti, F. G., L. J. Striker, M. A. Lesniak, K. MacKay, J. Roth, and G. E. Striker. 1988. Studies on binding and mitogenic effect of insulin and insulin-like growth factor I in glomerular mesangial cells. *Endocrinology*. 122:2788-2795.
10. Lovett, D. H., J. L. Ryan, and R. B. Sterzel. 1983. Stimulation of rat mesangial cell proliferation by macrophage interleukin 1. *J. Immunol.* 131:2830-2836.
11. Castellot, J. J., R. L. Hoover, M. J. Karnovsky. 1986. Glomerular endothelial cells secrete a heparinlike inhibitor and a peptide stimulator of mesangial cell proliferation. *Am. J. Pathol.* 125:493-500.
12. Castellot, J. J., R. L. Hoover, P. A. Harper, M. J. Karnovsky. 1985. Heparin and glomerular epithelial cell-secreted heparinlike species inhibit mesangial-cell proliferation. *Am. J. Pathol.* 120:427-435.
13. Roberts, A. B., M. B. Sporn. 1985. Transforming growth factors. *Cancer Surv.* 4:683-705.
14. Assoian, R. D., and M. B. Sporn. 1986. Type  $\beta$  transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* 102:1217-1223.
15. Roberts, A. B., M. A. Anzano, L. M. Wakefield, N. S. Roche, D. F. Stern, M. B. Sporn. 1985. Type  $\beta$  transforming growth factor: A bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA.* 82:119-123.
16. Majack, R. A. 1987. Beta-type transforming growth factor specifies organizational behavior in vascular smooth muscle cell cultures. *J. Cell Biol.* 105:465-471.
17. Masue, T., L. M. Wakefield, J. F. Lechner, M. A. LaVeck, M. B. Sporn, and C. C. Harris. 1986. Type  $\beta$  transforming growth factor is the primary differentiation-inducing factor for normal human bronchial epithelial cells. *Proc. Natl. Acad. Sci. USA.* 83:2438-2442.
18. Florini, J. R., A. B. Roberts, D. Z. Ewton, S. L. Falen, K. C. Flanders, and M. B. Sporn. 1986. Transforming growth factor- $\beta$ , A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by buffalo rat liver cells. *J. Biol. Chem.* 261:16509-16513.
19. Fine, A., and R. H. Goldstein. 1987. The effect of transforming growth factor- $\beta$  on cell proliferation and collagen formation by lung fibroblast. *J. Biol. Chem.* 262:3897-3902.
20. Centrella, M., T. L. McCarthy, and E. Canalis. 1987. Transforming growth factor  $\beta$  is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *J. Biol. Chem.* 262:2869-2874.
21. Ignatz, R. A., and J. Massague. 1986. Transforming growth factor- $\beta$  stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337-4345.
22. Roberts, A. B., M. A. Anzano, C. A. Meyers, J. Wideman, R. Blacher, Y.-C. E. Pan, S. Stein, S. R. Lehrman, J. M. Smith, L. C. Lamb, and M. B. Sporn. 1983. Purification and properties of a type beta transforming growth factor from bovine kidney. *Biochemistry*. 22:5692-5698.
23. Camussi, G., C. Tetta, G. Mazzucco, G. Monga, C. Roffinello, M. Alberton, P. Dellabona, F. Malavasi, and A. Vercellone. 1986. Platelet cationic proteins are present in glomeruli of lupus nephritis patients. *Kidney Int.* 30:555-565.
24. Duffus, P., A. Parbani, G. Frampton, and J. S. Cameron. 1982. Intraglomerular localization of platelet related antigens, platelet factor 4 and  $\beta$ -thromboglobulin in glomerulonephritis. *Clin. Nephrol.* 17:288-297.
25. Miller, K., I. G. Dresner, and A. F. Michael. 1980. Localization of platelet antigens in human kidney disease. *Kidney Int.* 18:472-477.
26. MacKay, K., L. J. Striker, S. Elliot, C. A. Pinkert, R. L. Brinster, and G. E. Striker. 1988. Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice. *Kidney Int.* 33:677-684.
27. Kenjaski, D., H. Poczewski, G. Dekan, R. Horvat, E. Balzar, N. Kraft, and R. C. Atkins. 1986. Identification of a major sialoprotein

in the glycocalyx of human visceral glomerular epithelial cells. *J. Clin. Invest.* 76:1142-1149.

28. Voyta, J. C., D. P. Via, C. E. Butterfield, and B. R. Zetter. 1984. Identification and isolation of endothelial cells based on their increased uptake of acetylated low density lipoprotein. *J. Cell Biol.* 99:2034-2040.

29. Conti, F. G., L. J. Striker, S. J. Elliot, D. Andreani, and G. E. Striker. 1988. Synthesis and release of insulinlike growth factor I by mesangial cells in culture. *Am. J. Physiol.* 255:F1214-F1219.

30. Wakefield, L. M., D. M. Smith, T. Masue, C. C. Harris, and M. B. Sporn. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J. Cell Biol.* 105:965-975.

31. Wakefield, L. M. 1987. An assay for type- $\beta$  transforming growth factor receptor. *Methods Enzymol.* 146:167-173.

32. Postlethwaite, A. E., G. N. Smith, C. L. Mainardi, J. M. Seyer, and A. H. Kang. 1984. Lymphocyte modulation of fibroblast function in vitro: stimulation and inhibition of collagen production by different effector molecules. *J. Immunol.* 132:2470-2477.

33. Olson, J. L. 1984. Role of heparin as a protective agent following reduction of renal mass. *Kidney Int.* 25:376-382.

34. Purkerson, M. L., P. E. Hollsten, and S. Klahr. 1976. Pathogenesis of the glomerulopathy associated with renal infarction in rats. *Kidney Int.* 9:407-417.

35. Klahr, S., M. L. Purkerson, and M. Heifets. 1987. Factors that may retard the progression of renal disease. *Kidney Int.* 32(Suppl.):S35-S39.

36. Donadio, J. V., C. F. Anderson, J. C. Mitchell, K. E. Holley, D. M. Ilstrup, V. Fuster, and J. H. Chesebro. 1984. Membranoproliferative glomerulonephritis. A prospective clinical trial of platelet-inhibitor therapy. *N. Engl. J. Med.* 310:1421-1426.

37. Assoian, R. K., B. E. Fleurdelys, H. C. Stevenson, P. J. Miller, D. K. Madtes, E. W. Raines, R. Ross, and M. B. Sporn. 1987. Expression and secretion of type  $\beta$  transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA.* 84:6020-6024.

38. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* 163:1037-1050.